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Novel gene delivery to liver cells using engineered virosomes

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Abstract We have demonstrated for the first time that the reconstituted Sendai viral envelopes containing only the fusion protein (F-virosomes) are efficient vehicles for the delivery of foreign genes specifically into human hepatoblastoma cells (HepG2) in culture. The membrane fusion-mediated entry of *CAT* (chloramphenicol acetyl transferase) gene into the cells was confirmed and the amount delivered to various subcellular fractions was quantitated. The dose dependence and kinetics of expression of biologically active CAT protein in HepG2 cells was measured. The CAT expression level in F-virosome-mediated delivery was significantly higher than that of Lipofectin or liganded proteo-liposome-mediated gene transfer. This kind of targeted delivery by means of membrane fusion induced by viral envelope glycoprotein may have wide applications to various gene transfer strategies both in vitro and in vivo.

Key words: Sendai virus; Virosome; Membrane fusion; Gene delivery; Gene therapy

1. Introduction

A major problem in the delivery of DNA and other biological macromolecules into cells is the crossing of the permeability barrier imposed by the plasma membrane. In the past few years, closed lipid bilayer vesicles (liposomes) have been used for delivering various macromolecules into living cells [1]. In this system the loaded liposomes are taken up by endocytosis and are released in lysosomes where low pH and various lysosomal enzymes inactivate the enclosed material [2]. Degradation of DNA in lysosomes is known to be one of the limiting factors in gene transfer by liposomes [3]. Although a recent study demonstrates the potential of cationic liposomes to transfer and express a human gene in the nasal epithelium of patients, the transfection efficiency and the duration of expression will need to be much improved from a wider therapeutic point of view [4]. Another delivery system is based on retroviral vectors either alone or in combination with cationic liposomes [5,6]. Although this mode has a higher transformation efficiency and exhibits stable transduction of non-dividing cells, the viral oncogene and the random insertion of the retroviral genes into the host genome have undesirable side effects besides inherent cytopathicity [7] and non-specific targeting to normal cells [8]. Adenovirus based gene transfer vectors suffer from similar problems [9,10].

Reconstituted Sendai viral envelopes (F,HN-virosomes) containing two glycoproteins, F (fusion protein) and HN (hemagglutinin-neuraminidase), are known to fuse efficiently with the plasma membrane of target cells and are excellent carriers for fusion-mediated microinjection of biologically active macromolecules in vitro [11]. This delivery system utilizes the binding of HN to the sialic acid residues of the membrane, followed by the F-protein-mediated fusion of the viral envelopes with the host cell plasma membrane at neutral pH. However, this promising system of gene delivery lacks cell type specificity because of the presence of HN protein, which is known to bind to various cell types through the sialic acid moiety of cell surface glycoconjugates. In spite of all recent developments in gene therapy since 1989, the formulation of a targeted gene delivery 'vector' is still far from ideal [8,12,13].

It has been recently demonstrated in our laboratory that F-virosomes (devoid of HN protein) can specifically bind and fuse with HepG2 cells [14]. The target specificity of F-virosomes has been ensured by the strong interaction between the terminal β-galactose moiety of F-protein and the asialoglycoprotein receptor (ASGP-R) on the membrane of HepG2 cells [14]. In further studies, F-virosomes have been successfully used for the delivery of biologically active macromolecules into the cytoplasm of liver cells both in vitro and in vivo [15–17]. Liver is known to be a model organ for somatic gene therapy. Hence, F-virosomes by virtue of their specific interaction and fusion with liver cells should be an ideal vector for gene delivery both in vitro and in vivo [18]. We report for the first time the F-virosome-mediated delivery of *CAT* gene and its expression in HepG2 cells in a systematic and quantitative fashion. The efficacy of this system for targeted gene expression in HepG2 cells is discussed.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strain DH5α was used for all transformation experiments. The plasmid pCIS2, containing the human cytomegalovirus (CMV) immediate early promoter-enhancer element, was obtained from Genentech, Inc., USA. Plasmid pTKCAT, containing the thymidine kinase promoter linked to the *CAT* gene was obtained from Dr. S.K. Goswami, State University of New York Health Science Center at Brooklyn, USA.

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Abbreviations: Ap, ampicillin; CAT, chloramphenicol acetyl transferase; CMV, cytomegalovirus; dhfr, dihydrofolate reductase; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; F, fusion protein; FCS, fetal calf serum; HN, hemagglutinin-neuraminidase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonylfluoride; RSVE, reconstituted Sendai viral envelope; RBC, red blood cell(s); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SV40, simian virus 40; TBS, Tris-buffered saline; TE, Tris-EDTA; WGA, wheat germ agglutinin

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2.2. Virus, cells

Sendai virus (Z strain) was grown, harvested, and purified following published protocols [14]. Viral yield was estimated in terms of protein and its activity was checked [14]. HepG2 cells and CHO (Chinese hamster ovary) cells were grown as described earlier [16].

2.3. Enzymes and chemicals

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs Inc., USA. Random primers DNA labeling system, Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), fetal calf serum (FCS), trypsin-EDTA, and Lipofectin® reagent were obtained from Life Technologies Inc., USA. CAT ELISA kit was purchased from Boehringer Mannheim, Germany. All other chemicals used were of the highest grade commercially available.

2.4. Construction and isolation of eukaryotic expression vector, pCIS3CAT

A 1.55 kb *Xho*1–*Sma*1 fragment from plasmid pTKCAT containing the *CAT* gene and SV40 polyadenylation signal was cloned into the plasmid pCIS2, downstream of the cytomegalovirus promoter–enhancer element. A putative clone was identified and confirmed by restriction mapping and Southern hybridization using ^{32}P-labeled *CAT* gene fragment as a probe [19]. This putative clone was designated as pCIS3CAT. The plasmid pCIS3CAT was isolated by standard alkaline lysis method and purified on CsCl density gradients [19].

2.5. Preparation of F-virosomes loaded with pCIS3CAT DNA

Reconstituted Sendai viral envelopes containing the F-protein (F-virosomes) were prepared as described [14]. The Triton X-100 solubilized fraction of virus was mixed with pCIS3CAT DNA (75 μg of DNA/mg of viral protein) and reconstituted by stepwise removal of detergent using SM2 biobeads. The unentrapped DNA adsorbed on outer surface of virosomal membrane was removed by treatment of virosomes with DNAase I (60 μg DNAase I/mg of F-protein) at 37°C for 30 min. The presence of entrapped DNA was checked by lysing virosomes with 2% SDS, loading on an 0.8% agarose gel, and subsequently staining with ethidium bromide. The amount of pCIS3CAT DNA entrapped in F-virosomes was calculated using ^{32}P-labeled DNA as a tracer. Structural integrity of loaded F-virosomes was checked for leakage of entrapped DNA during incubation with phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM phosphate, pH 7.4), with fresh mouse plasma and DMEM containing 10% FCS at 37°C for 16 h and after heat-treatment at 56°C for 30 min.

2.6. Fusion-mediated delivery of pCIS3CAT DNA to HepG2 cells and its quantitation in various subcellular fractions

The pCIS3CAT DNA was labeled with [α-^{32}P]dCTP by random primer labeling technique [19]. Monolayer cells (grown in T-25 flasks, 1×10^7 cells) were washed thrice with 2 ml of DMEM without serum. Cells were incubated with loaded F-virosomes (0.3 mg of F-protein containing 1 μg of labeled DNA, in 2 ml of DMEM without serum) for 2 h at 37°C/5% CO_2. HepG2 cells preincubated with 2 mg/ml asialofetuin, heat-treated F-virosomes and free DNA were used as controls. After 2 h of fusion, medium was replaced with DMEM containing serum (10% FCS) and cells were further incubated for 24 h at 37°C/5% CO_2. After 24 h, the cell surface bound (unfused) virosomes were removed by washing cells 4 times with 2 ml of ice-cold DPBS containing 5 mM EDTA [16]. After EDTA stripping, monolayer cells were lifted with 0.5 ml of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) and washed thrice with 1.5 ml of ice-cold Tris-buffered saline (TBS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4). Finally, the cell pellet was resuspended in 1 ml of isotonic homogenizing buffer (0.01 M Tris-HCl (pH 7.4) containing 0.25 M sucrose) and then dispersed in Potter-Elvehjem type homogeniser at 4°C for subcellular fractionation [16]. Plasmid DNA was extracted from each subcellular fraction in 1 M NaCl and 0.6% SDS according to Hirt lysis method [20]. DNA from each subcellular fraction was electrophoresed on 0.8% agarose gel and the bands corresponding to plasmid DNA and chromosomal DNA were cut from the dried gel and counts were measured. The amount of DNA internalized in the various subcellular fractions was determined from the specific activity of the ^{32}P-labeled DNA.

2.7. Expression of CAT gene in HepG2 cells

HepG2 cells were grown in monolayers in 6-well plates until a density of 2.5×10^5 cells. To monitor dose-dependent expression of CAT protein, loaded F-virosomes (0.3, 0.45, 1.2 and 3.0 mg of F-virosomes containing 1.0, 1.5, 4.0 and 10 μg of DNA, respectively), were incubated with HepG2 cells as described above. Lipofectin-mediated transfection of HepG2 cells with pCIS3CAT DNA was performed following a standard protocol [21]. To study time-dependent expression of CAT protein, F-virosomes (1.2 mg of F-virosomes containing 4 μg of pCIS3CAT DNA) were initially incubated with HepG2 cells for 2 h at 37°C/5% CO_2. After 2 h of fusion, medium was replaced as above and cells were incubated for varying times at 37°C/5% CO_2. In each of the above experiments, the amount of CAT protein expressed per milligram of cell protein was estimated by CAT ELISA (as suggested by the manufacturer) taking appropriate controls.

3. Results

3.1. Characterization of DNA loaded F-virosomes

F-virosome preparations were examined for purity by SDS-PAGE in the presence of β-mercaptoethanol [14] and were found to be free from any detectable contamination of other proteins. Membrane fusion activity of these virosomal preparations was checked by their ability to lyse mouse RBCs in the presence of WGA [14]. F-virosome associated pCIS3CAT DNA was DNAase I resistant, indicating that it was entrapped rather than adsorbed on the virosomal membrane (Fig. 1). No detectable leakage of DNA was observed from F-virosomes incubated with PBS or plasma/FCS and heat-treated F-virosomes. Two to 5 μg of intact DNA was found to be encapsulated in 1 mg of F-virosomes.

3.2. Uptake of pCIS3CAT DNA by HepG2 cells through membrane fusion and quantitation of DNA delivered to various subcellular fractions

F-virosome-mediated internalization of pCIS3CAT DNA by cells was examined prior to checking *CAT* gene expression. After 2 h of fusion followed by 24 h of incubation of HepG2 cells, the amount of plasmid DNA delivered by F-virosomes

Table 1
Specificity of F-virosome-mediated DNA delivery

Treatment	Amount of ^{32}P-labeled DNA internalized (μg)/mg of cell protein		Amount of CAT protein expressed (pg)/mg of cell protein	
	HepG2 cells	CHO cells	HepG2 cells	CHO cells
F-virosomes	0.5	0.03	275.00	[a]
F-virosomes+2 mg/ml asialofetuin	0.04	[a]	30.00	[a]

HepG2 cells and CHO cells were incubated with loaded F-virosomes (1 μg of ^{32}P-labeled DNA, 0.3 mg of F-protein) in the presence or absence of asialofetuin at the specified concentration, for 2 h at 37°C and were further incubated for 24 h as described in the text. After 24 h of incubation, cell extracts were assayed for radioactivity and in a parallel set of experiments, the amount of CAT protein expressed per milligram of cell protein was determined. The values are average of two independent determinations.

[a] Not detected.

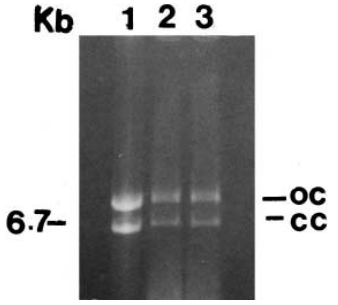

Fig. 1. Agarose gel electrophoresis of pCIS3CAT DNA entrapped in F-virosomes: F-virosome sample (containing 70 μg of F-protein) was lysed with 2% SDS, loaded on an 0.8% agarose gel and run at 100 V for 1 h. The bands were visualized after staining the gel with ethidium bromide. Lane 1: Free pCIS3CAT DNA (1 μg); Lane 2: Entrapped pCIS3CAT DNA before DNAase I treatment; Lane 3: Entrapped pCIS3CAT DNA after DNAase I treatment.

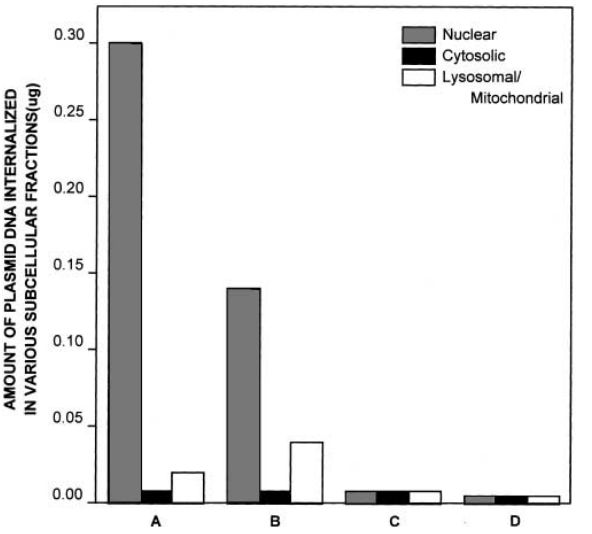

Fig. 2. Subcellular distribution of ^{32}P-labeled pCIS3CAT DNA delivered to HepG2 cells by F-virosomes: HepG2 cells were incubated with F-virosomes loaded with ^{32}P-labeled DNA and the corresponding controls as described in the text. Subcellular fractions were processed for DNA isolation by Hirt lysis and were electrophoresed on 0.8% agarose gel. The plasmid bands cut from the dried gel were assayed for radioactivity. The values are mean of duplicate determinations. A,B: Subcellular fractions of HepG2 cells incubated with F-virosomes and heat-treated F-virosomes, respectively, containing 1 μg of DNA. C: Subcellular fractions of HepG2 cells incubated with 2 mg/ml asialofetuin prior to the addition of F-virosomes loaded with 1 μg of DNA. D: Subcellular fractions of HepG2 cells incubated with 1 μg of free plasmid DNA.

(containing 1 μg of DNA; Fig. 2), was found to be maximum in the nuclear fraction. However, no radioactivity was detected from the chromosomal DNA bands, thereby ruling out the possibility of integration of the plasmid in the host DNA. The amount of DNA delivered to nucleus by the corresponding heat-treated F-virosomes was 2 times less than that delivered by untreated F-virosomes. Heat-treated F-virosomes being fusion-inactive [16], are likely to be taken up by endocytosis leading to their accumulation in lysosomes. This results in significant degradation of pCIS3CAT DNA, as shown by a very low level of DNA detected in the lysosomal/mitochondrial fraction (Fig. 2). The binding of F-virosomes to liver cells is known to be strongly inhibited in the presence of asialofetuin [17]. This was found to be consistent in our case as loaded untreated and heat-treated F-virosomes failed to transfer any detectable level of DNA to HepG2 cells preincubated with asialofetuin (Table 1). Under similar conditions, loaded F-virosomes were unable to deliver pCIS3CAT DNA to CHO cells (Table 1) that lack the ASGP-R [22]. These results strongly support the target specific, fusion-mediated delivery of pCIS3CAT DNA by F-virosomes and efficient transport of intact DNA to the nuclear compartment of HepG2 cells.

3.3. *Expression of CAT gene in HepG2 cells after fusion-mediated DNA delivery*

The *CAT* gene expression in HepG2 cells was examined as a function of DNA dose upon fusion-mediated delivery by F-virosomes. The maximum amount of CAT protein detected was achieved with 4 μg of DNA loaded in F-virosomes (Fig. 3A). The amount of CAT protein expressed after F-virosome-mediated delivery was 3–4 times more than that of the corresponding heat controls in all doses of DNA. HepG2 cells preincubated with asialofetuin and CHO cells (with or without asialofetuin preincubation), did not exhibit any detectable CAT expression (Table 1). In case of free DNA incubated with HepG2 cells and cells alone, no significant ELISA signal was detected (data not shown). These data suggest the targeted and efficient gene expression through F-virosomes. The presence of serum (10% FCS) during fusion (initial 2 h incubation) of loaded F-virosomes with HepG2 cells resulted in a modest difference in CAT expression when compared to fusion in absence of serum (Table 2). It is also interesting to note that 1 μg of pCIS3CAT DNA delivered by the Lipofectin method of transfection did not express any detectable level of CAT protein. However, 15 μg of DNA delivered by Lipofectin transfection expressed 300 pg of CAT protein which is comparable to the protein expressed (0.06 fg of CAT protein/cell) when 1 μg of DNA was delivered by F-virosomes (Fig. 3B). The amount of CAT protein expressed at varying times after fusion-mediated delivery of DNA was evaluated to check the stability of this transduction process. The amount of CAT protein expressed was found to be persistent till a period of 192 h (Fig. 4), thereby indicating the overall superior efficiency of this delivery system.

Table 2
Effect of serum on F-virosome-mediated DNA delivery and *CAT* gene expression

Treatment	Amount of CAT protein expressed (pg)/mg of cell protein	
	+Serum	−Serum
pCIS3CAT loaded F-virosomes	440 ± 38.1	533.3 ± 32.8
CIS3CAT loaded F-virosomes (heated at 56°C, 30 min)	106.6 ± 8.0	88.0 ± 7.62

HepG2 cells were incubated with loaded F-virosomes (containing 4 μg of DNA and 1.2 mg of F-protein) in DMEM with and without serum (10% FCS). The corresponding heat-treated F-virosomes were used as controls. Values represent mean (± SD) of three independent determinations.

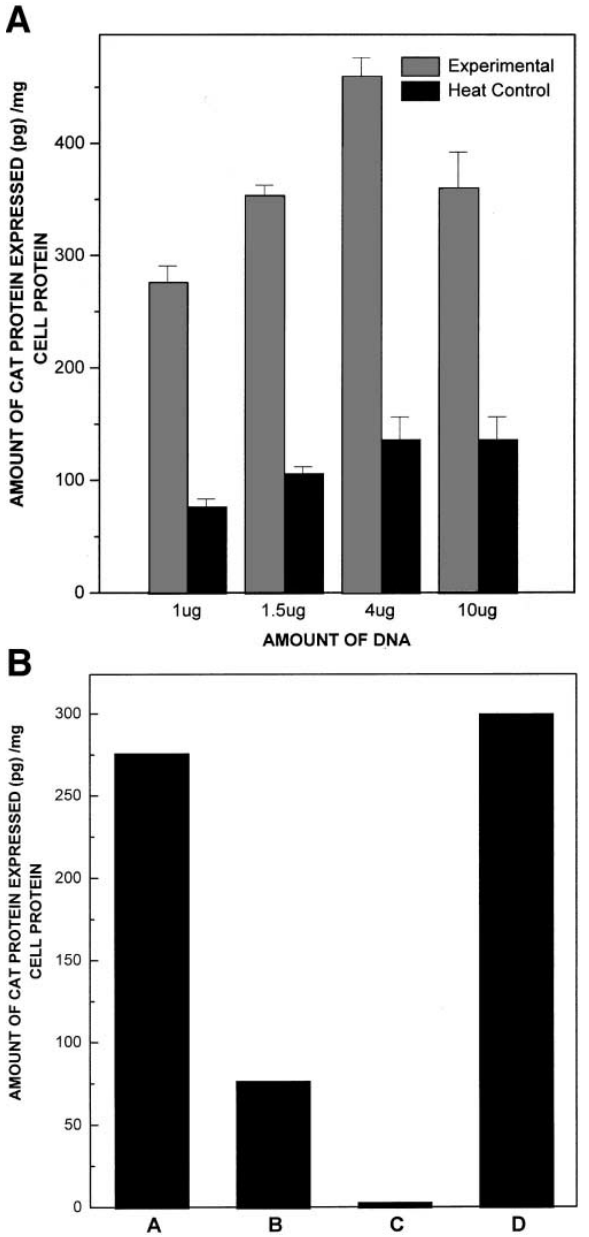

Fig. 3. A: Dose-dependent expression of pCIS3CAT DNA delivered to HepG2 cells by F-virosomes: HepG2 cells were incubated with loaded F-virosomes containing varying amounts of DNA and the corresponding heat controls (details in the text). The amount of CAT protein expressed per milligram of cell protein was determined. Each value is mean (±SD) of three independent determinations. B: Comparison of F-virosome, proteo-liposome and Lipofectin-mediated DNA delivery systems: HepG2 cells were incubated with untreated and heat-treated F-virosomes containing 1 μg of pCIS3-CAT DNA as in (A). The values are mean of duplicate determinations. A,B: Amount of CAT protein expressed after delivery of 1 μg of DNA by F-virosomes and heat-treated F-virosomes, respectively. C,D: Amount of CAT protein expressed after delivery of 1 μg and 15 μg of DNA by Lipofectin.

4. Discussion

The present findings constitute a rational and quantitative approach to targeted delivery and expression of a foreign reporter gene (*CAT*) in liver cells using a novel vehicle derived from Sendai viral envelopes (F-virosomes), with the added novelty of avoiding the degradation of the entrapped DNA caused by the endocytotic pathway. We have recently established in our laboratory that F-protein behaves both as a ligand and membrane fusogen for targeting of virosomal aqueous contents to the cytosolic compartment of liver cells both in vitro and in vivo [15–17]. It was shown previously that heat-treatment of F-protein in F-virosomes completely abrogates its fusogenic potential without significantly affecting the galactose-mediated specific recognition of ASGP-R [16]. This has suggested the use of heat-treated F-virosomes loaded with DNA, as liganded proteo-liposomes, which may be efficiently endocytosed by HepG2 cells. In Fig. 2, the endocytotic pathway has been compared with that of fusion-mediated delivery of DNA by F-virosomes. The amount of DNA delivered to the nuclear compartment of HepG2 cells by fusion mode is appreciably more than that by heat-treated F-virosomes. This confirms the efficient delivery of DNA to the nucleus of HepG2 cells incubated with F-virosomes as compared to their heat-treated counterparts (Fig. 2). Considering the results from other appropriate controls (Fig. 2 and Table 1) of these experiments, the specificity and the increased efficiency of this DNA delivery vehicle in terms of nuclear transfer is clearly apprehended. Further evidence of fusion-mediated delivery of pCIS3CAT DNA comes from the quantitation of dose-dependent (Fig. 3A) and time-dependent (Fig. 4) expression of CAT protein in HepG2 cells. The expression of CAT has been compared with that of other conventional delivery methods (Fig. 3A,B). The heat-treated F-virosomes are found to be inefficient in terms of CAT expression, presumably because of degradation of a major fraction of DNA in the endocytotic vesicles of HepG2 cells. This conforms to the DNA delivery process as shown in Fig. 2. Recently, adenovirus modified with asialoglycoprotein-polylysine conjugate was used to enhance targeted gene expression in liver cells through receptor-mediated endocytosis in vitro [23]. Besides the risk of cytopathic side effects of adenovirus based delivery systems (which

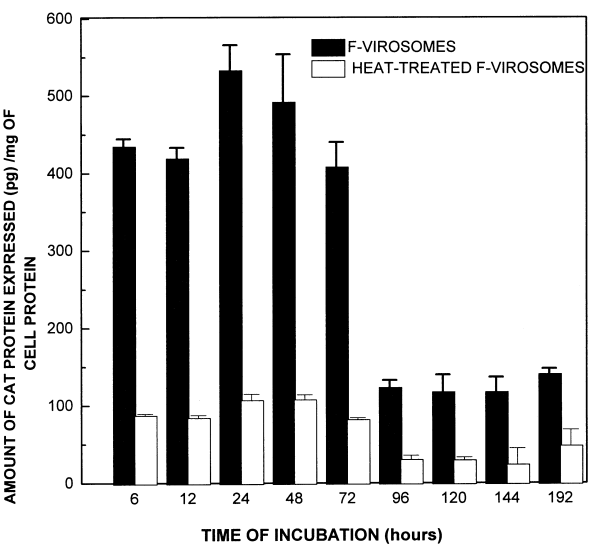

Fig. 4. Kinetics of expression of pCIS3CAT DNA delivered to HepG2 cells by F-virosomes: HepG2 cells (2.5×10^5) were incubated with untreated and heat-treated loaded F-virosomes containing 4 μg of DNA, for varying times at 37°C. At the indicated time points, cell extracts were prepared and CAT ELISA was performed as mentioned in the text. The values represent mean (±SD) of three independent determinations.

limits its in vivo applications) the efficiency of the system in terms of transgene expression was 3–4 times less than that of F-virosomal delivery. The difference between the F-virosomal delivery system and Lipofectin-mediated transfection is more striking (Fig. 3B). In F-virosome mediated delivery, 1 μg of encapsulated DNA is sufficient for detectable expression of CAT, whereas 15 μg of DNA is required in the Lipofectin mode. HepG2 cells incubated with free pCIS3CAT DNA fail to express any detectable CAT protein, showing thereby the efficiency of this F-virosomal delivery system. In addition, the persistent expression of CAT protein at a significant level in vitro (Fig. 4) may be considered as an unique feature of this fusion mode of gene delivery. Moreover, serum-insensitivity of this delivery system in terms of *CAT* gene expression strongly supports its potential as an effective DNA carrier for in vivo application (Table 2). The probable reason of this efficient and persistent expression could be attributed to the delivery of large amount of functional pCIS3CAT DNA into the nucleus of HepG2 cells. Considering the high levels of CAT production achieved in vitro, we conclude that this delivery vehicle has immense potential for targeted delivery of nucleic acids of therapeutic importance in the field of gene therapy.

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